Lack of Direct Association between *EGFR* Mutations and ER Beta Expression in Lung Cancer

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Abstract. *Background: Positive expression of estrogen receptor (ER) beta is correlated with a favorable prognosis for patients with epidermal growth factor receptor (EGFR) mutations and predicts a good clinical outcome for patients with lung cancer after treatment with an EGFR-tyrosine kinase inhibitor (TKI), suggesting that it may be a candidate surrogate marker. The molecular mechanism underlying the apparent link between EGFR mutations and ER beta expression in lung cancer cell lines was investigated. Materials and Methods: Four different human lung cancer cell lines were used, including one with an exon19 delE746-A750, one with a substitution of Leu for Arg at codon 858 in exon 21 (L858R), one with a L858R+ threonine-to-methionine mutation at codon 790 of EGFR (T790M) and one with wild-type EGFR. The EGFR mutations were investigated by direct sequencing. The expression levels of ER beta in the cell lines, in tumors from SCID mice and in primary human tissue specimens were evaluated by immunohistochemistry. Cell growth was compared after treatment with 17-beta-estradiol. The proliferative activity following knockdown of ER beta by siRNA was also examined. Furthermore, the cell inhibition assay was performed for cells treated with gefitinib after knockdown of ER beta. To investigate the relevant pathways for ER beta, the expression of apoptosis-related molecules was evaluated by Western blotting analysis. Results: All the cell lines showed positive expression of ER beta, the cancer cells from SCID mice, and the original primary tumors showed positive expression of ER beta. All of the cell lines revealed a similar proliferative pattern, regardless of the presence of EGFR mutations. Although the suppression of ER beta slightly increased the proliferative activity, no statistically significant effect on proliferation was observed in any of the cell lines. Moreover, no significant changes in any cell signaling or apoptosis-related molecules were observed following ER beta knockdown. Conclusion: A direct association between EGFR mutations and ER beta expression in lung cancer cell lines is lacking. Further investigation will be necessary to clarify the role of the ER in the EGFR signaling pathway.*

Lung cancer continues to be the leading cause of cancer mortality in both males and females throughout the world (1). Female gender has been confirmed to be an independent favorable prognostic indicator for non-small cell lung cancer (NSCLC) survival (2), and observations by the Women's Health Initiative trial strongly suggested that hormone replacement therapy (HRT) provides a tumor growth advantage in lung cancer, suggesting an estrogen receptor (ER) role in carcinogenesis (3).

Interactions between the ER and the epidermal growth factor receptor (EGFR) contribute to the biological effects of these binding protein families (4). *EGFR* mutations occur more frequently in adenocarcinoma of the lung with positive expression of ER beta, a subtype of ER (5). We have previously demonstrated positive expression of ER beta to be associated with a favorable prognosis (5), which was consistent with previous findings (6-8), and to predict a good clinical outcome for lung adenocarcinoma patients who were treated with EGFR-tyrosine kinase inhibitors (TKIs), thus suggesting that may represent a good candidate surrogate marker (9). However, it is not known whether ER beta has a direct functional role in the response to EGFR-TKIs. Whether or not the expression of ER beta affects the sensitivity of human lung cancer cells to EGFR-TKIs was examined. The relevant pathway underlying the potential signaling pathway for ER beta was also investigated by Western blotting analysis. This was the first analysis of the relationship between EGFR-activating mutations and ER beta expression in lung cancer cell lines performed to elucidate the mechanism underlying their apparent relationship.

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Materials and Methods

Cell culture. Human lung cancer cell lines L804L, B901L and G603L were established in our laboratory from primary tissue specimens (10). The NSCLC cell line H1975 was purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in RPMI growth medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum at 37˚C and 5% CO_2. The 17-beta-estradiol (E_2) was purchased from Sigma-Aldrich Japan (E2758, Tokyo, Japan). Gefitinib was provided by AstraZeneca (Macclesfield, UK).

Detection of EGFR mutations. The genomic DNA was extracted from each of the cell lines and the *EGFR* mutations were examined by previously described methods (11). The Institutional Review Board approved the use of the tumor specimens that were obtained with the informed consent of either the patients or their legal guardians.

Immunohistochemical staining for ER beta. The ER beta immunohistochemical (IHC) staining was carried out by previously described methods (5, 9). Briefly, the primary antibody reaction used an anti-ER beta Ab (H-150; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:50 in PBS and incubated for 18 h at 4˚C. Thereafter, IHC staining was performed by the labeled polymer method (Histofine Simple Stain MAX-PO kit; Nichirei, Tokyo, Japan) according to the manufacturer's instructions. The IHC staining was evaluated by a previously defined scoring method (9).

Engraftment of human lung cancer cells into SCID mice. Female severe combined immunodeficiency (SCID) mice (6 weeks old) were purchased from Charles River Japan (Tokyo, Japan) and were maintained under specific pathogen-free conditions throughout the study. Approximately 5×10^6 fresh lung cancer cells were injected under the skin of the SCID mice. The tumors were obtained after 2 weeks of growth.

Detection of ER beta mRNA. The ER beta gene copy numbers were analyzed by previously described methods (12) and involved a quantitative real-time PCR, performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a Fast SYBR Green Master Mix (Applied Biosystems). Each DNA sample from the cultured cells and tumors was quantified by comparing the target locus to β-actin as an internal control. The primer sequences of the ER beta gene for quantitative real-time PCR were ATAGCCCTG CTGTGATGAATTACA and GTTGGCCACAACACA TTTGG. Those for beta-actin were TCCTTCCTGGGTAGGTGTTG and GATGCTGTGTCACCGAGGAT. The quantification was based on the standard curves from a serial dilution of the human normal lung DNA. PCR was performed for each primer set in triplicate and the mean value was calculated.

Knockdown analysis using small-interfering RNA (siRNAs). The following double-stranded RNA 25 base pair oligonucleotides for ER beta were synthesized by Stealth Select RNAi (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions; #1: 5'-AAUGCUGUAAUUCAUCACAGCAGGG-3' and 5'-CCCUGCUG UGAUGAAUUACAGCAUU-3', #2: 5'-AGGUGUUGGCCACAAC ACAUUUGGG-3' and 5'-CCCAAAUGUGUUGUGGCCAACAC CU-3', #3: 5'-AUAACUGGCGAUGGACCACUAAAGG-3' and 5'-CCUUUAGUGGUCCAUCGCCAGUUAU-3'. The control siRNA was the commercially available stealth RNAi negative control medium GC duplex (Invitrogen). The siRNA transfections were performed according to the manufacturer's instructions. Ten microliters of lipofectamine 2000 (Invitrogen) were diluted in 250 ml Opti-MEM I medium (Invitrogen) and incubated for 5 min at room temperature. Next, 250 pmol of ER beta inverted control duplex Stealth RNA (Invitrogen) or one of the three oligonucleotides diluted in 250 ml Opti-MEM I were added gently and the mixture incubated for 20 min at room temperature. The oligomer–lipofectamine complexes and aliquots of 3×10^5 cells in 500 ml culture medium were combined and incubated for 10 min at room temperature. The cells were seeded in 6-well clear plates (Corning, Lowell, MA,USA) and harvested after 72 h in culture to determine the cell count and for the Western blotting analyses. In addition, 5×10^3 of the cells were seeded in 96-well clear plates (Corning) and cultured with or without gefitinib, then the cell viability of the *EGFR* mutants was examined. The quantification of cell number was performed in triplicate and the mean value and standard deviation (SD) was calculated.

Cell inhibition assay. The cell viability was examined using a cell counting kit-F, which is a fluorometric assay based on cell lysis and staining methods (Wako, Tokyo, Japan) by previously described methods (13). Following incubation with or without gefitinib, the cells were washed twice with 100 μl of PBS and incubated with 7.5 μmol Calcein-AM solution in PBS at 37˚C for 30 min. The fluorescence intensity was measured with a 485 nm excitation and a 535 nm emission filter using an SH-1000Lab instrument (Corona Electric, Ibaraki, Japan). The quantification was performed in triplicate, and the mean value and SD was calculated.

Western blotting analyses. After the lung cancer cell lines were transfected with siRNA against ER beta or a control siRNA, and cultured for 72 h, whole-cell lysates (100 μg) were loaded onto polyacrylamide gels in Tris-glycine SDS running buffer (SDS–PAGE) as described previously (14). The proteins were transferred to a polyvinylidine difluoride microporous membrane (Millipore, Bedford, MA, USA) using a semi-dry blotter. The blotted membrane was treated with 5% skimmed milk in 10 mM Tris, 150 mM NaCl and 0.2% Tween 20 and incubated at 4˚C overnight with a 1:1000 dilution of antibodies against including molecules related to cell signaling or the apoptosis pathway (15), cyclin D1, cyclin D3, CDK4, CDK6, $p15^{INK4B}$, $p16^{INK4A}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$, cleaved caspase 9, caspase 3, cleaved caspase 3, caspase 7, cleaved caspase 7, PARP, and cleaved PARP (Cell Signaling Technology, Danvers, MA, USA). The membrane was then incubated for 60 min at room temperature with secondary anti-mouse or anti-rabbit IgG antibody, conjugated with horseradish peroxidase. Immunoblots were enhanced by Lumi-Lightplus Western blotting substrate (Roche, IN, USA).

Statistical analysis. Statistical associations were determined by *t*-tests. The statistical difference was considered to be significant if the *p*-value was <0. 05. The data were analyzed with the use of the Abacus Concepts, Survival Tools for Stat View software package (Abacus Concepts, Inc., CA, USA).

Results

The EGFR mutations were first examined in nine cell lines, and from them, four different EGFR mutant cell lines which showed positive expression of ER beta were selected. (10,

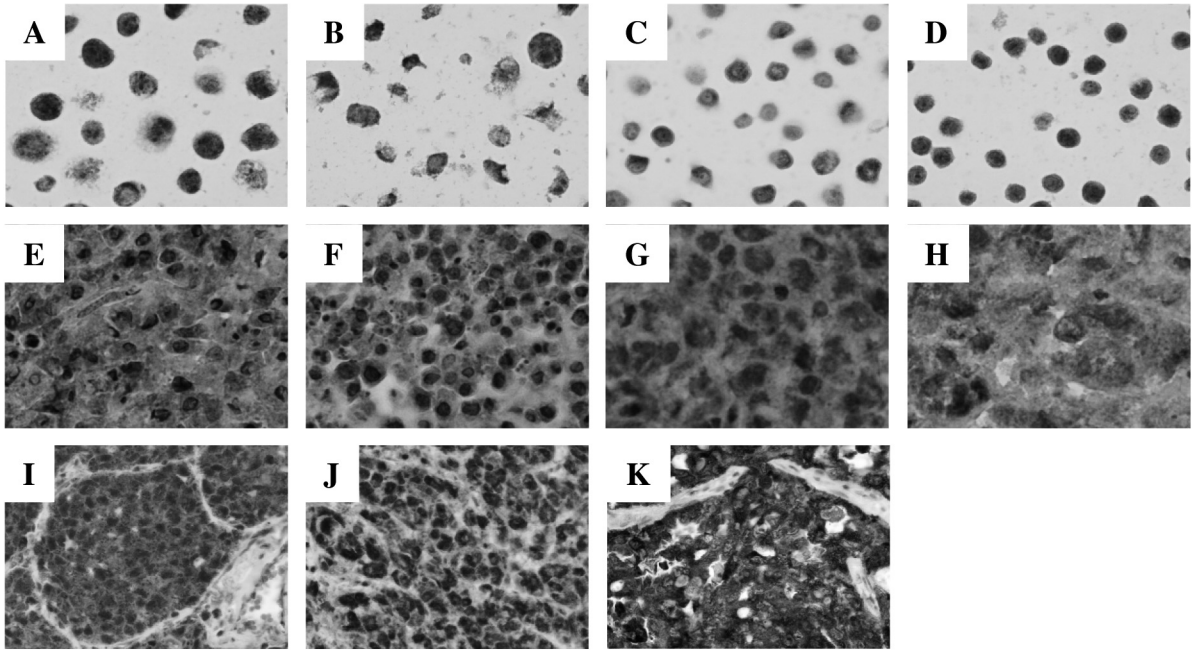

Figure 1. *Immunohistochemical staining. Positive expression of ER beta in cultured cells (original magnification ×400). A: B901L, B: G603L, C: H1975, D: L804L; indicated by brown staining in cells from SCID mice. E: B901L, F: G603L, G: H1975, H: L804L and in the primary tumor from which I: B901L. J: G603L and K: L804L cells were derived.*

13) (Table I). All the cell lines including EGFR-TKI-resistant H1975 cells (13) showed positive expression of ER beta in the cultured cells, cancer cells grown in SCID mice, and in the original primary tumor specimens (Figure 1).

After adding E_2, all the cell lines revealed a similar proliferative pattern, regardless of the type of *EGFR* mutation (Figure 2A). Following siRNA knockdown of endogenous ER beta, all three oligonucleotides led to similar down-regulation of ER beta mRNA (Figure 2B).

Although there was a tendency toward a slight increase in the proliferative activity after knockdown for ER beta, no statistically significant change in growth was observed in any of the cell lines using representative siRNA#2 (Figure 2C). Furthermore, cell proliferation was also not affected by knockdown for ER beta following treatment with gefitinib in any of the cell lines using representative siRNA#2 (Figure 2D). By Western blotting analysis, the ER beta status was not significantly related to any cell signaling or apoptosis-related molecules (Figure 3).

Table I. *Lung cancer cell lines and EGFR status.*

Cell line	Histology	*EGFR* mutation
B901L	Adenocarcinoma	Exon19 delE746-A750
G603L	Pleomorphic	Exon21 L858R
H1975	Adenocarcinoma	Exon21 L858R+Exon20 T790M
L804L	Adenocarcinoma	Wild-type

Discussion

The present study demonstrated three major findings. First, the lung cancer cells expressing ER beta showed augmented proliferation when treated with E_2, which was consistent with other studies (16, 17). However, there were no significant differences in the growth or proliferation of different *EGFR* mutant cells after treatment with E_2, suggesting no crosstalk between mutant EGFR and ER beta in response to E_2.

Second, suppression of ER beta expression by siRNA slightly (but not significantly) increased the proliferative activity of the cells. Zhang *et al*. reported that an ER beta-specific ligand promoted the growth of lung cancer cells and that knockdown of ER beta resulted in the loss of estrogen-dependent growth of lung cancer cells (18). In fact, ER beta might act as a tumor suppressor in opposition to ER alpha (19). Interestingly, a previous study showed that ER-beta could protect tumor cells from undergoing aggressive epithelial-mesenchymal transition (EMT) (20), which plays a part in determining the sensitivity to EGFR- TKIs (21). On the other hand, treatment with E_2 led to an ER beta-mediated inhibition of malignant mesothelioma cell proliferation, as well as up-regulation of p21 and p27 (22). Thus, there might be origin-specific effects with regard to those molecules.

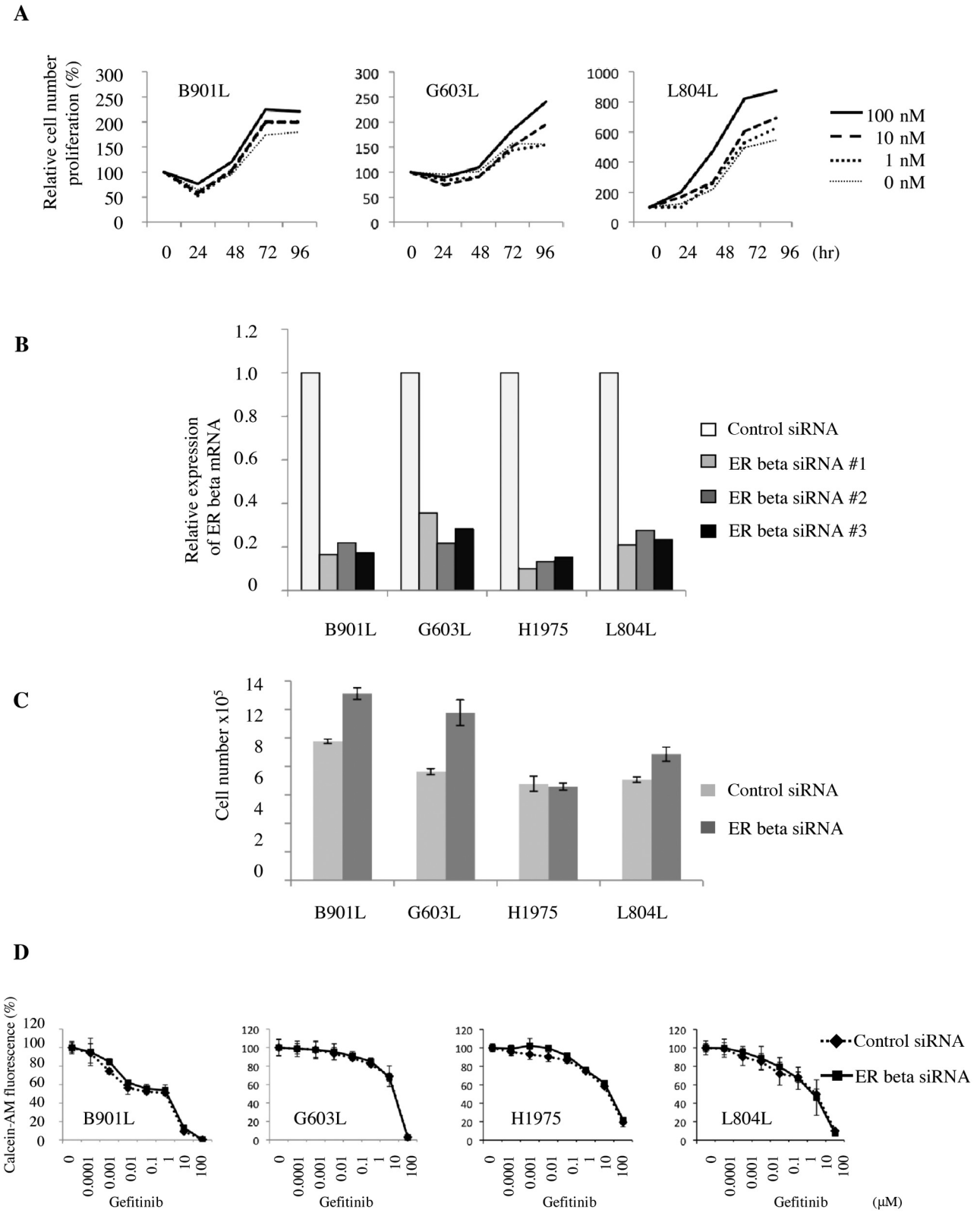

Figure 2. *A: The time course of mean number of cancer cells after exposure to E_2. B: Relative expression of ER beta mRNA after knockdown of ER beta. Mean value of quantifications in triplicate. C: The mean value and SD of cancer cell number after knockdown of ER beta: D: Cell inhibition after knockdown of ER beta and treatment with or without gefitinib.*

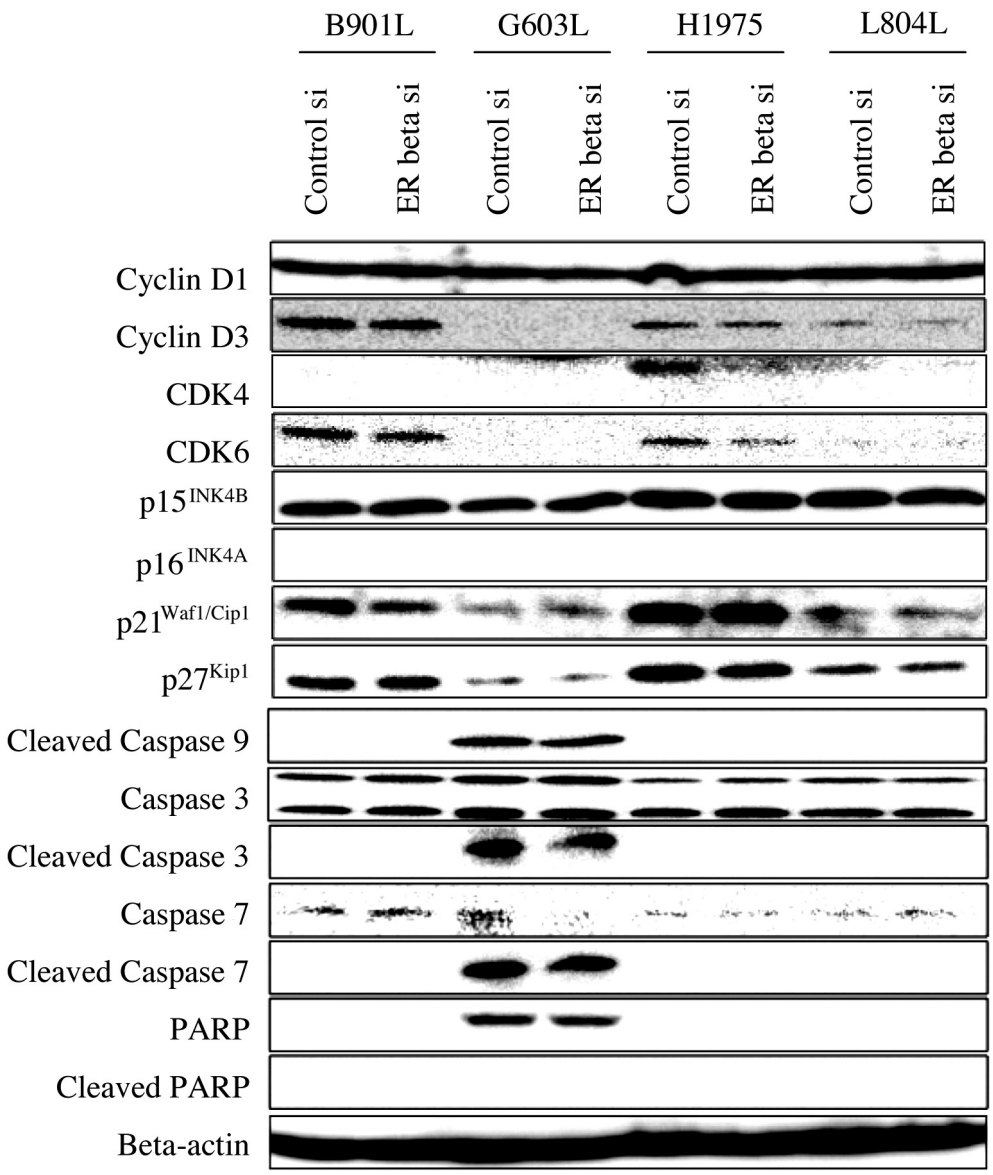

Figure 3. *Western blot analysis using antibodies for cyclin D1, cyclin D3, CDK4, CDK6, $p15^{INK4B}$, $p16^{INK4A}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$, cleaved caspase 9, caspase 3, cleaved caspase 3, caspase 7, cleaved caspase 7, PARP and cleaved PARP.*

Wheter the hypothesis that the ER beta expression could reflect the sensitivity of cells to EGFR-TKIs was tested using siRNA for ER beta, unexpectedly, no statistically significant inhibition cell growth was observed in any of the cell lines. This finding suggested that ER beta expression does not determine the sensitivity of cells to EGFR-TKIs *in vitro*. The discrepancies between the clinical specimens (5) and the current *in vitro* study may be attributable to the techniques used to establish the cell lines, as well as differences in sampling, the microenvironment (including the presence of fibroblasts and macrophages) and other unknown mechanisms. Stabile *et al*. reported that the EGFR pathway is activated when estrogen is depleted in lung cancer cells (15), since all the cell lines expressed ER beta, this may have been a possible limitation in the current study.

In conclusion, a direct association between *EGFR* mutations and ER beta expression in lung cancer cell lines is lacking. However, evidence accumulated over the past decade describes cross-talk between the ER and EGFR in the clinical setting (4). Further translational research is therefore needed in the future to clarify the role of ER in the EGFR signaling pathway.

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